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# UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

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| First Named Inventor or Application Identifier | | | |
| Ari HINKKANEN | | | |
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**APPLICATION ELEMENTS**

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: **Assistant Commissioner of Patents**
**Box Patent Application**
**Washington, D.C. 20231**

1. [X] Fee Transmittal Form
   *(Submit an original, and a duplicate for fee processing)*
2. [X] Specification Total pages [22]
   *(preferred arrangement set forth below)*
   - Descriptive title of the invention
   - Cross references to Related Applications
   - Statement Regarding Fed sponsored R&D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings
   - Detailed Description
   - Claims
   - Abstract of the Disclosure
3. [X] Drawing(s) (35 USC 113) (Total Sheets) [12]
4. [X] Oath or Declaration (Total Pages) [2 ]
   a. [X] Newly executed (original or copy)
   b. [ ] Copy from a prior application (37 CFR 1.63(d)
   (for continuation/divisional with Box 17 completed)
   ***[Note Box 5 below]***
   i [ ] DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)
5. [ ] Incorporation by Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. [ ] Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
   a. [ ] Computer Readable Copy
   b. [ ] Paper Copy (identical to computer copy)
   c. [ ] Statement verifying identity of above copies

**ACCOMPANYING APPLICATION PARTS**

8. [ ] Assignment Papers (cover sheet & document(s))
9. [ ] 37 CFR 3.73(b) Statement
   *(when there is an assignee)*
   [X] Associate Power of Attorney
10. [ ] English Translation Document *(if applicable)*
11. [ ] Information Disclosure Statement /PTO 1449
    [ ] Copies of IDS Citations
12. [X] Preliminary Amendment
13. [X] Return Receipt Postcard (MPEP 503)
    *(Should be specifically itemized)*
14. [ ] Small Entity Statement(s)
    [ ] Statement Filed in prior application, Status still proper and desired
15. [ ] Certified Copy of Priority Document(s).
    *(if foreign priority is claimed)*
16. [ ] Other:

17. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*

[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application No.:

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[ ] Customer Number or Bar Code Label or [ X ] Correspondence address below
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| | | | | | |
|---|---|---|---|---|---|
| *Name* | Jeffrey L. Ihnen, Reg. No. 28,957 Jeffrey Ihnen | | | | |
| *Address* | Rothwell, Figg, Ernst & Kurz<br>Suite 701-East, 555 13th Street, N.W. | | | | |
| *City* | Washington | *State* | D.C. | *Zip Code* | 20004 |
| *Country* | U.S.A. | *Telephone* | 202-783-6040 | *Fax* | 202-783-6031 |

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| In re Application of | ) | |
| Ari HINKKANEN | ) | |
| Serial No. (to be assigned) | ) | Examiner: |
| Filed: 29 January 1998 | ) | Group Art Unit: |
| For: A NEW FUSION PROTEIN AND ITS USE IN AN IMMUNOASSAY FOR THE SIMULTANEOUS DETECTION OF AUTOANTIBODIES RELATED TO INSULIN-DEPENDENT DIABETES MELLITUS | ) | |

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to initial examination of the above application, filed concurrently herewith, please enter the following amendments:

IN THE ABSTRACT:

At the top of the unnumbered page containing the Abstract, please insert -- 22 --.

IN THE CLAIMS:

Please amend the claims as follows:

In claim 9, line 1, delete "or 8".

Please add the following new claim:

--17. A vector comprising the cDNA according to claim 8.--

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## REMARKS

The above amendments to the claims are to delete a multiple dependency, and bring the claims more in line with U.S. practice. The amendment to the Abstract is merely to insert an identifying page number.

It is believed that these amendments do not constitute the insertion of new matter. Prompt consideration of this Preliminary Amendment is requested.

Respectfully submitted,

By Jeffrey L. Ihnen

Jeffrey L. Ihnen
Attorney for Applicants
Registration No. 28,957

ROTHWELL, FIGG, ERNST & KURZ, p.c.
Suite 701-E, 555 13th Street, N.W.
Washington, D.C. 20004
Telephone: (202)783-6040

Dated: 29 January 1998

# A NEW FUSION PROTEIN AND ITS USE IN AN IMMUNOASSAY FOR THE SIMULTANEOUS DETECTION OF AUTOANTIBODIES RELATED TO INSULIN-DEPENDENT DIABETES MELLITUS

## FIELD OF THE INVENTION

This invention relates to a new fusion protein, its cDNA, and a vector and a cell comprising said cDNA. Furthermore, this invention relates to the use of said fusion protein in an immunoassay for simultaneous detection of autoantibodies related to insulin dependent diabetes mellitus.

## BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

GAD65, IA2 and insulin are pancreatic proteins produced by the beta cells (for review see Atkinson and Maclaren 1993). Autoantibodies to these proteins are detected in patients with insulin-dependent diabetes mellitus (IDDM) and healthy individuals at risk for developing the disease. More than 80 % of newly-diagnosed IDDM patients have antibodies against at least one of these proteins (Baekkeskov et al. 1982). The risk of diabetes in relatives of IDDM patients increases markedly when the number of autoantibodies detected in the serum increases (Bingley et al. 1994; Verge et al. 1994). In a group of high genetic risk, presence in serum of antibodies to one or more of these autoantigens predicted the disease onset accurately (Verge et al. 1996). Also permanently healthy subjects (as regards IDDM) may have temporarily or permanently antibodies against one of the three antigens, but antibodies against multiple antigens occur extremely rarely. It is therefore sought to simultaneously determine reactivity against two or all three of the proteins, as the positivity for more than one

of these autoantibodies remarkably increases disease risk (Bingley et al. 1994).

GAD65 (Bu et al. 1992) has several epitopes recognised by autoantibodies (Falorni et al. 1996). These are located mostly at the center and C-terminus of the molecule whereas the N-terminal quarter of the molecule is thought to contribute to membrane docking of the protein, and to contain few if any IDDM-informative epitopes (Falorni et al. 1996).

IA2 (also known as ICA512) (Rabin et al. 1994) is a transmembrane protein with still unknown function. The intracellular part of the molecule ($IA2_{ic}$, about 40 kDa) contains a domain with similarity to the active center of protein phosphatases (Fischer et al. 1991), but no enzymatic activity has been ascribed the IA2 molecule. The informative epitopes of IA2 reside in the cytoplasmic domain and herein they are concentrated at the C-terminal half (Lampasona et al. 1996; Zhang et al. 1997).

Insulin (Bell et al. 1980) is made by pancreatic β-cells as a precursor preproinsulin which is cleaved to proinsulin. The proinsulin is further processed to give the insulin consisting of A and B chains connected together with two disulphide bridges.

More than 20% of sera collected from newly-diagnosed IDDM-patients contain insulin autoantibodies (IAA) (Sabbah et al. 1996). As, however, the immunity to insulin may have arisen through formation of response to prepro- or proinsulins (Snorgaard et al. 1996), it is relevant to use these peptides in this assay system. Tolerance to this autoantigen may be induced by oral insulin feeding in non-obese diabetic (NOD) mice (Zhang et al. 1991).

In addition to linear epitopes, autoantibodies are thought to recognize important conformational epitopes resulting

from the three-dimensional structure of the protein (Kim et al. 1993). Antigen molecules produced or assayed using techniques which destroy these structures are less informative as regards IDDM or prediabetes.

Several methods for detection of autoantibodies in IDDM sera have been elaborated. One method exploits in vitro transcription-translation for producing radioactively labeled autoantigen (IA2, GAD65) (Petersen et al. 1994), while in another method biotin-labeled GAD65 is added to the patient sera and after formation of immune complexes, free label is detected and quantitated (Mehta et al. 1996). These methods all suffer from suboptimal niveau of informativity, as they employ only one specific autoantigen. Moreover they have the drawbacks associated with the use of radiochemicals.

Using a protein molecule in which a combination of the epitopes from at least two but preferably three different autoantigens are represented should detect a larger panel of autoantibodies thus revealing more specifically the population of individuals at risk of developing the disease.

SUMMARY OF THE INVENTION

According to one aspect, this invention relates to a new fusion protein having epitopes of at least two of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS) wherein said epitopes are connected with a linker peptide, said fusion protein being able to bind to a solid phase.

According to another aspect, the invention concerns a cDNA sequence encoding the said fusion protein.

According to a third aspect, the invention concerns a vector and a cell comprising said cDNA.

According to a fourth aspect, the invention concerns an immunoassay for the simultaneous determination in a sample of a person's body fluid of at least two insulin-dependent diabetes mellitus (IDDM) -related autoantibodies, wherein each autoantibody is specific for an epitope of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) or preproinsulin (PPINS). The immunoassay comprises the steps of

- incubating said sample with said autoantigens or, alternatively, with the fusion protein according to this invention, said autoantigens or said fusion protein being bound to a solid support,
- adding at least one labeled reagent capable of binding to one or more of said autoantibodies, and
- quantifying the signals from the labels bound to the solid phase.

According to still one aspect, the invention concerns a method for diagnosing a person's risk of developing insulin-dependent diabetes mellitus (IDDM), said method comprising the determination in a sample of said person's body fluid of at least two insulin dependent diabetes mellitus (IDDM) -related autoantibodies specific for an epitope of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) or preproinsulin (PPINS), wherein the presence of at least two of said autoantibodies are indicative for said person's risk of developing IDDM. The order of appearance of these autoantibodies is used to predict the time point of onset of the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b show the cDNA construct for a fusion protein according to this invention,

Figure 2a shows the amino acid sequence of the IA2 protein,

Figure 2b shows the amino acid sequence of the GAD65

protein,

Figure 2c shows the amino acid sequence of preproinsulin (PPINS),

Figures 3a-3b show the nucleotide sequence encoding GAD65,

Figures 3c-3e show the nucleotide sequence encoding IA2,

Figures 3f-3i show the human insulin gene,

Figure 4 shows the fusion protein according to this invention attached to a solid support, autoantibodies attached to epitopes of said protein, and labeled reagents bound to said autoantibodies, wherein the reagents are labeled with different labels, and

Figure 5 shows the fusion protein according to this invention attached to a solid support, autoantibodies attached to epitopes of said protein, and labeled reagents bound to said autoantibodies, wherein the reagents are labeled with the same label.

DETAILED DESCRIPTION OF THE INVENTION

The term "epitope" can be an amino acid sequence anything from very few (about 5 to 10) amino acids of the autoantigens up to the whole autoantigen. Preferable lengths of the epitopes are represented by the underlined amino acid sequences in Figures 2a and 2b, and the whole antigen sequence is disclosed in Figure 2c. Thus, the epitope of IA2 comprises preferably the amino acids 771-979 of the amino acid sequence shown in Figure 2a. Another preferred alternative is the whole intracellular domain (amino acids ranging from about 576 to 979 of the sequence in Figure 2a). The epitope of GAD65 comprises preferably the amino acids 102-585 of the amino acid sequence shown in Figure 2b, and the epitope of PPINS comprises preferably

all the amino acids 1-110 of the polypeptide shown in Figure 2c. It should be noted that the above mentioned specific sequences are examples only.

According to a preferred embodiment, the fusion protein has epitopes of each of the autoantigens GAD65, IA2 and PPINS. Such a fusion protein allows simultaneous detection of autoantibodies specific for any of said autoantigens.

Said fusion protein containing epitopes of GAD65, IA2 and PPINS is formed by combining these domains via short peptides consisting of amino acid residues, e.g. lysine and arginine residues.

The epitopes from distinct autoantigens will be linked together via short peptides containing e.g. several lysine residues, which allows preferential labeling of these lys-residues. For construction of the polygenic cDNA, the linker-encoding cDNA contains a recognition site for a rarely cutting restriction enzyme such as Not I or Sgf I (see Figure 1a and 1b).

These linker residues may be connected to a member of an affinity binding pair so as to enable the binding of said fusion protein to a solid phase. The bioaffinity pair may be e.g. biotin - streptavidin. The residues (lysine) can be biotinylated after which the fusion protein is attached to a streptavidin-coated solid phase. The solid phase can e.g. be a well of a microtitration strip or plate. Alternatively, the solid phase consists of microparticles.

The fusion protein can alternatively be bound to the solid phase by direct adsorption. Furthermore, the fusion protein can be covalently linked to the solid phase. In this case the fusion protein must be provided with groups able to create a covalent bond with the solid phase.
Figures 2 and 3 show the amino acid sequences and the nucleotide sequences, respectively, of the preferred

epitopes.

The following illustrates the construction of the fusion protein and its preparation.

The N-terminus of the hybrid protein will contain a flag peptide NH2-DYKDDDDK-COOH with a free N-terminal amino group to allow recognition of the protein using M1 monoclonal antibody (ATCC cell line nr. HB 9259). This enables detection of the protein in SDS-PAGE where not all monoclonals function.

At the carboxy-terminal end of the fusion protein and in the single antigens a motif X-X-G-S-H-H-H-H-H-H is introduced to allow purification of the protein with metal chelate affinity chromatography and detection with monoclonal antibody against this epitope (Cedarlane Laboratories Ltd, Canada).

The GAD65 gene (Bu et al. 1992) is, for example, amplified with PCR (nucleotides 1311-1755) in such a manner that 101 amino acid residues are removed from the N-terminus.

The 3'-end oligonucleotide contains 17 bases complementary to the mRNA of GAD65 and an additional sequence encoding half of a peptide forming the bridge between GAD65 and IA2 domains.

The nucleotide sequence of the bridge is for example

Not I

GAD65-AAGAAGAAGCGGCCGCGAAAGAAGAAG-IA2 (amino acid sequence of the peptide KKKRPRKKK), or

Sfg I

GAD65-AAGAAGAAGCGATCGCGAAAGAAGAAG-IA2 (amino acid sequence KKKRSRKKK). The restriction enzyme recognition sites are underlined in the middle. The fragments are made from a

plasmid harbouring said cDNAs with PCR and digested with appropriate restriction enzymes (e.g. Not I or Sfg I) and cloned into appropriate vectors. The GAD65 part is linked to IA2 and this to PPINS, using general cloning techniques.

The PPINS gene 5'-oligo contains half of the polylysine-arginine-encoding sequence with a Not I or Sfg I site for coupling to the IA2 gene 3'-end. The 3'-oligo of PPINS has a histidine hexapeptide-encoding sequence to enable antibody recognition and metal chelate chromatography purification and/or immobilization if necessary (Mauch et al. 1993).

Purified, restriction enzyme-treated PCR fragments are cloned in a FastBac derivative and E.coli DH10Bac cells are transfected with the plasmid. Recombinant clones are selected and DNA isolated and transfected into Sf9 insect cells.

Virus-producing cells are cultivated and stock virus made. Large-scale cultures are used to produce recombinant single proteins and the polyprotein.

SDS-PAGE/Western analysis is used to analyse size and immunoreactivity of the recombinant polyproteins. The proteins are blotted onto a nitrocellulose or nylon membrane and GAD/IA2/PPINS antibodies used to detect the product visualised with enhanced chemiluminescence, ECL.

For purification of the polyprotein GAD65-specific monoclonal antibody (GAD6, Developmental Studies Hybridoma Bank, Iowa University) is immobilized to Sepharose 4B activated with cyanogen bromide (Pharmacia, Uppsala, Sweden). Elution of the protein is performed at low pH (3-4) and solubility is achieved by adding detergents (e.g. Nonidet or Tween) to allow dissociation from the membranes.

The steps from cloning to large scale production can be

described in more detail as follows:

1. Cloning into the pK503-9 vector (Kari Keinänen VTT Finland), a derivative of pFastBac (Gibco BRL Paisley Scotland) of GAD65, or IA2 or PPINS gene, each containing a flag recognition signal (FLAG[R], Immunex Corporation) for antibody detection and a signal peptide for ecdysone glucotransferase (EGT) for transport into the endoplasmatic reticulum for removal of the signal peptide with simultaneous release of N-terminal aspartate for M1 antibody recognition. The constructs contain each a X-X-G-S-H-H-H-H-H-H carboxyterminal peptide to allow metal chelate affinity purification and detection with specific antibody (Cedarlane, Canada) of the product.

2. Transformation into competent E. coli DH10Bac cells of the plasmids containing the single genes.

3. Isolation of recombinant Bacmid DNA and transfection with the fused DNA of the Sf9 or Hi-5 insect cells.

4. Production of recombinant stock virus.

5. Large scale production of the proteins.

6. Cloning into pK503-9 vector of a cDNA construct for the fusion protein (FP) comprising GAD65 (nt 1311-1755; aa 102-585)-IA2(nt 2313-2937; aa 771-979)-PPINS (nt 2424-2610 and 3396-3539 (of the genomic DNA sequence, accession No. V00565); aa 1-110) in all alternative orders.

7. Transformation into competent E. coli DH10Bac cells of the plasmids containing the fusion protein.

8. Isolation of recombinant Bacmid DNA and transfection with the fused DNA of the Sf9 or Hi-5 insect cells.

9. Production of recombinant stock virus.

10. Large scale production of the fusion protein.

In case the baculovirus expression system does not work optimally, alternative systems such as E.coli, yeast, or in vitro transcription translation assay (Petersen et al. 1994) will be used for production of said polypeptides.

The present invention relates further to the use of the fusion protein in an immunoassay for the detection of several pancreatic beta-cell autoantibodies in IDDM patients and prediabetic sera. The assay may detect patients at risk of developing IDDM, i.e. having a pre-IDDM condition. As a multicomponent assay, the method could also be used to predict the time point of onset of the disease. The methodology which combines epitopes of several islet beta cell autoantigens increases the informativity and prediction value of the test aimed at prediction of risk and onset of disease in individuals genetically predisposed to IDDM.

In the immunoassay according to this invention, a sample of the person's body fluid (e.g. serum) is incubated with the fusion protein bound to a solid surface, e.g. a microtitration plate. The bound autoantigens are thereafter detected with a labeled reagent. The reagents can be the single autoantigens GAD65, IA2 and PPINS; or proteins comprising epitopes thereof. These reagents are used to detect free antigen-binding regions (V-regions) on the bound autoantibodies. One variant of the method will be used for differential detection of the individual autoantigen specificities of the antibody in one assay if individual autoantigens (AAGs) labeled with three different labels are used (see Figure 4). Alternatively, when the polyprotein (the fusion protein) is labeled with only one label, it can be used to reveal the sum of these three reactivities in the sample (Figure 5). The same result is achieved if the single antigens are all labeled with the same label. The labeled reagent can further be an anti-

human monoclonal antibody. In this case the assay can reveal only the sum of the three autoantibodies.

The technique which involves use of the label attached to the fusion protein or individual autoantigens circumvents several problems encountered in the conventional assays. First, there is little or no nonspecific binding to the vials due to the fact that the carrier surfaces have already been blocked with the corresponding antigen. Second, the attachment via a bioaffinity pair such as streptavidin/biotin interaction to the vial and use of a flexible peptide between the individual antigenic epitopes enable free motion and folding of the protein in the solution (Figure 5).

The label can be any suitable label. However, according to a preferred embodiment, the label is a lanthanide. In case three different labels are used, said labels can be e.g. Eu, Sm, Tb and Dy (Siitari et al. 1990; Hemmilä et al. 1993). In such a case the detection is based on time-resolved fluorescence.

The free labeled reagent can be removed after the incubation step before the signal is quantified (heterogeneous assay), or the signal can be quantified without foregoing removal of the free labelled reagent (homogeneous assay).

The procedures are preferably automatized. Automatization of the procedures involves laboratory robots which apply samples onto cover slips and the fluorescence is detected in an micro array system in an appropriate unit (Wallac OY, Finland).

The simultaneous detection of antibodies against the three autoantigens increases the capacity to process large sample series. The use of a micro array system substantially increases the capacity. This has become necessary as

nationwide screenings of newborns are undertaken in several research centers.

The test principle using time-resolved fluoroimmunoassay (TR-FIA) offers an extremely sensitive means for detection of autoantibodies with minimum amount of nonspecific reactivity due to used specific antigen label. The longevity of the lanthanide label is also an advantage as compared to radiolabel.

The system allows retaining of important conformational epitopes of the antigen as immobilization of the polyprotein is via specific flexible intervening sequences and causes minimal tortion to the antigen.

The following illustrates the use of the fusion protein in an immunoassay:

To the polyprotein (fusion protein) biotin is bound in limiting conditions to prevent other than the lysine residues of the linker peptide to be biotinylated. Streptavidine-coated microscope slides are treated with biotin - fusion protein and the residual sites are blocked with bovine serum albumin or another suitable binding protein.

M1 flag-specific monoclonal antibody will be used to monitor binding onto solid support of free recombinant autoantigens while autoantigen-specific monoclonals (e.g. GAD1, GAD6, MICA-3 (Boehringer) etc.) will be used to detect availability of specific epitopes. After incubation with sample sera, Eu-labeled GAD65, Sm-labeled IA2 and Tb-labeled PPINS (produced as a single protein with the baculosystem) are printed robotically onto the microscope slides in four quadrants covering an area of about 1 $cm^2$, allowed to bind, washed and dried in vacuum, and the fluorescence is measured on TR fluorometer.

The functionality of the method is tested using IDDM sera known to be positive for one or more of the antigens used.

For specificity testing recombinant GAD65, IA2 and PPINS, or fusion protein are added into patient sample to preadsorb specific antibodies.

The informativity will be compared with conventional systems. Statistical tests will be used to create best possible segregation of the positive and negative assay values.

The high density array system is fully automatized.

The invention is further illustrated by the following examples.

Example 1

**Labeling procedure**

Isothiocyantophenyl-DTTA-Eu, or Tb, or Sm (Mukkala 1989) will be used for labeling of the FP or the single autoantigens. Mainly the protocols of Lövgren & Petterson (1990) and Hemmilä et al. (1984) will be followed. 30-100 fold molar excess of the label substance will be used giving approximately 10-12 lanthanide molecules per protein molecule. For Tb, 500 fold excess will be used. The coupling is carried out for 18 hr at 0 °C in 0.1 M bicarbonate buffer pH 9.2. The Eu (Tb,Sm)-AAg complex is separated from free Eu (Tb, Sm) by gel filtration in a Sepharose 6B column equilibrated with 0.05 M Tris-HCl buffer pH 7.75 containing 0.9% NaCl and 0.05% $NaN_3$. The Eu-AAg complex is stored at 4 °C.

Example 2

**Immunoassay**

The assay is performed in the wells of polystyrene microtitration strip coated with unlabeled autoantigen preparate for 18 hr at 25 °C in 0.1 M bicarbonate buffer pH 9.6 (Siitari & Kurppa 1987). The strips are washed prior to use with 0.9% NaCl containing 0.05 % Tween 20 and 0.3% Germal II. To each well 100 µl of diluted (1:10) serum is added and incubated for 1 hr at 40 °C, washed 2x with the wash solution and 200 µl of the Eu-labeled autoantigen fraction (50 ng/well) is added.

The strips are incubated for 1 hr at 40 °C. The strips are washed 5x with the washing solution. Thereafter Enhancement Solution (EG&G Wallac) 200 µl/well is added. Strips are shaken for 10 min in a plate shaker and measured in EG&G Wallac Victor fluorometer for 1s/specimen. The photons emitted are measured as counts/s. Automated data reduction program calculates mean value of duplicates and the coefficient of variation (CV%).

For future development, the assay formate will be miniaturized e.g. by immobilizing the autoantigen molecules onto microparticles (Lövgren et al. 1997) or as a microarray onto glass cover slips.

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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CLAIMS

1. A fusion protein having epitopes of at least two of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS) wherein said epitopes are connected with a linker peptide, said fusion protein being able to bind to a solid phase.

2. The fusion protein according to claim 1 having epitopes of each of the autoantigens GAD65, IA2 and PPINS.

3. The fusion protein according to claim 2 wherein
- the epitope of IA2 comprises the amino acids 771-979 of the amino acid sequence shown in Figure 2a,
- the epitope of GAD65 comprises the amino acids 102-585 of the amino acid sequence shown in Figure 2b, and
- the epitope of PPINS comprises all the amino acids 1-110 of the amino acid sequence shown in Figure 2c.

4. The fusion protein according to claim 1 wherein the linker peptide comprises lysine and argine residues.

5. The fusion protein according to claim 4 wherein said linker peptide is provided with a member of an affinity binding pair so as to enable the binding of said fusion protein to the solid phase.

6. The fusion protein according to claim 5 wherein the affinity binding pair is biotin - streptavidin.

7. A cDNA encoding the fusion protein according to claim 1 wherein said cDNA comprises the nucleotide sequences encoding the epitopes of at least two of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS).

8. A cDNA encoding the fusion protein according to claim 3

wherein said cDNA comprises the nucleotide sequences a) nucleotides 1311 to 1755 of the sequence according to Figures 3a to 3b encoding GAD65, aa 102-585, b) nucleotides 2313 to 2937 of the sequence according to Figures 3c to 3e encoding IA2, aa 771-979, and c) nucleotides 2424 to 2610 and 3397 to 3539 of the sequence according to Figure 3f-3i encoding PPINS, aa 1-110, where said nucleotide sequences a), b) and c) can appear in any relative order.

a 9. A vector comprising the cDNA according to claim 7 ~~or 8.~~

10. An E. coli cell encompassing the cDNA according to claim 7.

11. An immunoassay for the simultaneous determination in a sample of a person's body fluid of at least two insulin dependent diabetes mellitus (IDDM) related autoantibodies, wherein each autoantibody is specific for an epitope of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) or preproinsulin (PPINS), said immunoassay comprising the steps of
- incubating said sample with a fusion protein according to claim 1, said fusion protein being bound to a solid support,
- adding at least one labeled reagent capable of binding to one or more of said autoantibodies, and
- quantifying the signals from the labels bound to the solid phase.

12. The immunoassay according to claim 11 wherein the labeled reagent is an anti-human monoclonal antibody.

13. The immunoassay according to claim 11 wherein the labeled reagent comprises at least two antigens labeled with different labels, each antigen being one of the autoantigens GAD65, IA2 or PPINS; or proteins comprising epitopes thereof.

14. The immunoassay according to claim 11 wherein the labeled reagent comprises three antigens labeled with the same label, each antigen being one of the autoantigens GAD65, IA2 or PPINS; or proteins comprising epitopes thereof.

15. The immunoassay according to claim 11 wherein the label is a fluorescent lanthanide chelate.

16. A method for diagnosing a person's risk of developing insulin dependent diabetes mellitus (IDDM), said method comprising the determination in a sample of said person's body fluid of at least two insulin dependent diabetes mellitus (IDDM) related autoantibodies specific for an epitope of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) or preproinsulin (PPINS), wherein the presence of at least two of said autoantibodies are indicative for said person's risk of developing IDDM.

(57) ABSTRACT

The invention relates to a fusion protein having epitopes of at least two of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS) wherein said epitopes are connected with a linker peptide. The fusion protein must be able to bind to a solid phase.

The invention also concerns the cDNA, and a vector and cell comprising said cDNA. Furthermore, this invention relates to the use of said fusion protein in an immunoassay for the simultaneous detection of autoantibodies related to insulin-dependent diabetes mellitus.

```
Flag-peptide        GAD65           Sgf I           IA2          Sgf I       PPINS      poly-his
DYKDDDDK--------------------KKKRPRKKK----------------- KKKRPRKKK ----------CNGSHHHHHH
```

FIG. 1a

```
Flag-peptide        GAD65           Not I           IA2          Not I       PPINS      poly-his
DYKDDDDK-------------------- KKKRSRKKK ------------------ KKKRSRKKK----------CNGSHHHHHH
```

FIG. 1b

IA2 Underlined aa 771-979 Accession No. L18983

MRRPRRPGGLGGSGGLRLLLCLLLLSSRPGGCSAVSAHGCLFDRRLCSHLEVCIQDGLFGQCQVGVGQARPLLQVTSPVLQRL
QGVLRQLMSQGLSWHDDLTQYVISQEMERIPRLRPPEPRPRDRSGLAPKRPGPAGELLLQDIPTGSAPAAQHRLPQPPVGKGG
AGASSSLSPLQAELLPPLLEHLLLPPQPPHPSLSYEPALLQPYLFHQFGSRDGSRVSEGSPGMVSVGPLPKAEAPALFSRTASKGI
FGDHPGHSYGDLPGPSPAQLFQDSGLLYLAQELPAPSRARVPRLPEQGSSSRAEDSPEGYEKEGLGDRGEKPASPAVQPDAAL
QRLAAVLAGYGVELRQLTPEQLSTLLTLLQLLPKGAGRNPGGVVNVGADIKKTMEGPVEGRDTAELPARTSPMPGHPTASPT
SSEVQQVPSPVSSEPPKAARPPVTPVLLEKKSPLGQSQPTVAGQPSARPAAEEYGYIVTDQKPLSLAAGVKLLEILAEHVHMSS
GSFINISVVGPALTFRIRHNEQNLSLADVTQQAGLVKSELEAQTGLQILQTGVGQREEAAAVLPQTAHSTSPMRSVLLTLVALA
GVAGLLVALAVALCVRQHARQQDKERLAALGPEGAHGDTTFEYQDLCRQHMATKSLFNRAEGPPEPSRVSSVSSQFSDAAQ
ARIKLKVESSPSRSDYINASPIIEHDPRMPAYIATQGPLSHTIADFWQMVWESGCTVIVMLTPLVEDGVKQCDRYWPDEGASLY
HVYEVNLVSEHIWCEDFLVRSFYLKNVQTQETRTLTQFHFLSWPAEGTPASTRPLLDFRRKVNKCYRGRSCPIIVHCSDGAGR
TGTYILIDMVLNRMAKGVKEIDIAATLEHVRDQRPGLVRSKDQFEFALTAVAEEVNAILKALPQ

FIG. 2a

GAD65 Underlined aa102-585 Accession No. M74826

MASPGSGFWSFGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYGDAEKPAESGGSQPPRAAARKAACACDQKPCS
CSKVDVNYAFLHATDLLPACDGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELADQPQNLEEILMHC
QTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTANTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAIS
NMYAMMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAALGIGTDSVILIKCDERGKMIPSDLERRILEAKQKGFVPF
LVSATAGTTVYGAFDPLLAVADICKKYKIWMHVDAAWGGGLLMSRKHKWKLSGVERANSVTWNPHKMMGVPLQCSALLV
REEGLMQNCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHVDVFKLWLMWRAKGTTGFEAHVDKCLELAEYLYNIIKNR
EGYEMVFDGKPQHTNVCFWYIPPSLRTLEDNEERMSRLSKVAPVIKARMMEYGTTMVSYQPLGDKVNFFRMVISNPAATHQ
DIDFLIEEIERLGQDL

FIG. 2b

Translation Human preproinsulin.
EMBL accession nr. v00565

MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYT
PKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQ
LENYCN

FIG. 2c

**Human GAD65 nucleotide sequence**

M74826 Length: 2457 September 1, 1995 12:22 Type: N Check: 8038 ..



1 ACCCGCCCTC GCCGCTCGGC CCCGCGCGTC CCCGCGCGTG CCCTCCTCCC
51 GCCACACGGC ACGCACGCGC GCGCAGGGCC AAGCCGAGGC AGCCGCCCGC
101 AGCTCGCACT CGCTGGCGAC CTGCTCCAGT CTCCAAAGCC GATGGCATCT
151 CCGGGCTCTG GCTTTTGGTC TTTCGGGTCG GAAGATGGCT CTGGGGATTC
201 CGAGAATCCC GGCACAGCGC GAGCCTGGTG CCAAGTGGCT CAGAAGTTCA
251 CGGGCGGCAT CGGAAACAAA CTGTGCGCCC TGCTCTACGG AGACGCCGAG
301 AAGCCGGCGG AGAGCGGCGG GAGCCAACCC CCGCGGGCCG CCGCCCGGAA
351 GGCCGCCTGC GCCTGCGACC AGAAGCCCTG CAGCTGCTCC AAAGTGGATG
401 TCAACTACGC GTTTCTCCAT GCAACAGACC TGCTGCCGGC GTGTGATGGA

451 GAAAGGCCCA CTTTGGCGTT TCTGCAAGAT GTTATGAACA TTTTACTTCA
501 GTATGTGGTG AAAAGTTTCG ATAGATCAAC CAAAGTGATT GATTTCCATT
551 ATCCTAATGA GCTTCTCCAA GAATATAATT GGGAATTGGC AGACCAACCA
601 CAAAATTTGG AGGAAATTTT GATGCATTGC CAAACAACTC TAAAATATGC
651 AATTAAAACA GGGCATCCTA GATACTTCAA TCAACTTTCT ACTGGTTTGG
701 ATATGGTTGG ATTAGCAGCA GACTGGCTGA CATCAACAGC AAATACTAAC
751 ATGTTCACCT ATGAAATTGC TCCAGTATTT GTGCTTTTGG AATATGTCAC
801 ACTAAAGAAA ATGAGAGAAA TCATTGGCTG GCCAGGGGGC TCTGGCGATG
851 GGATATTTTC TCCCGGTGGC GCCATATCTA ACATGTATGC CATGATGATC
901 GCACGCTTTA AGATGTTCCC AGAAGTCAAG GAGAAAGGAA TGGCTGCTCT
951 TCCCAGGCTC ATTGCCTTCA CGTCTGAACA TAGTCATTTT TCTCTCAAGA
1001 AGGGAGCTGC AGCCTTAGGG ATTGGAACAG ACAGCGTGAT TCTGATTAAA
1051 TGTGATGAGA GAGGGAAAAT GATTCCATCT GATCTTGAAA GAAGGATTCT
1101 TGAAGCCAAA CAGAAAGGGT TTGTTCCTTT CCTCGTGAGT GCCACAGCTG
1151 GAACCACCGT GTACGGAGCA TTTGACCCCC TCTTAGCTGT CGCTGACATT
1201 TGCAAAAAGT ATAAGATCTG GATGCATGTG GATGCAGCTT GGGGTGGGGG
1251 ATTACTGATG TCCCGAAAAC ACAAGTGGAA ACTGAGTGGC GTGGAGAGGG

FIG. 3a

1301 CCAACTCTGT GACGTGGAAT CCACACAAGA TGATGGGAGT CCCTTTGCAG

1351 TGCTCTGCTC TCCTGGTTAG AGAAGAGGGA TTGATGCAGA ATTGCAACCA

1401 AATGCATGCC TCCTACCTCT TTCAGCAAGA TAAACATTAT GACCTGTCCT

1451 ATGACACTGG AGACAAGGCC TTACAGTGCG GACGCCACGT TGATGTTTTT

1501 AAACTATGGC TGATGTGGAG GGCAAAGGGG ACTACCGGGT TTGAAGCGCA

1551 TGTTGATAAA TGTTTGGAGT TGGCAGAGTA TTTATACAAC ATCATAAAAA

1601 ACCGAGAAGG ATATGAGATG GTGTTTGATG GGAAGCCTCA GCACACAAAT

1651 GTCTGCTTCT GGTACATTCC TCCAAGCTTG CGTACTCTGG AAGACAATGA

1701 AGAGAGAATG AGTCGCCTCT CGAAGGTGGC TCCAGTGATT AAAGCCAGAA

1751 TGATGGAGTA TGGAACCACA ATGGTCAGCT ACCAACCCTT GGGAGACAAG

1801 GTCAATTTCT TCCGCATGGT CATCTCAAAC CCAGCGGCAA CTCACCAAGA

1851 CATTGACTTC CTGATTGAAG AAATAGAACG CCTTGGACAA GATTTATAAT

1901 AACCTTGCTC ACCAAGCTGT TCCACTTCTC TAGAGAACAT GCCCTCAGCT

1951 AAGCCCCCTA CTGAGAAACT TCCTTTGAGA ATTGTGCGAC TTCACAAAAT

2001 GCAAGGTGAA CACCACTTTG TCTCTGAGAA CAGACGTTAC CAATTATGGA

2051 GTGTCACCAG CTGCCAAAAT CGTAGGTGTT GGCTCTGCTG GTCACTGGAG

2101 TAGTTGCTAC TCTTCAGAAT ATGGACAAAG AAGGCACAGG TGTAAATATA

2151 GTAGCAGGAT GAGGAACCTC AAACTGGGTA TCATTTGCAC GTGCTCTTCT

2201 GTTCTCAAAT GCTAAATGCA AACACTGTGT ATTTATTAGT TAGGTGTGCC

2251 AAACTACCGT TCCCAAATTG GTGTTTCTGA ATGACATCAA CATTCCCCCA

2301 ACATTACTCC ATTACTAAAG ACAGAAAAAA ATAAAAACAT AAAATATACA

2351 AACATGTGGC AACCTGTTCT TCCTACCAAA TATAAACTTG TGTATGATCC

2401 AAGTATTTTA TCTGTGTTGT CTCTCTAAAC CCAAATAAAT GTGTAAATGT

2451 GGACACA

FIG. 3b

## Human IA-2 nucleotide sequence

L18983 Length: 3613 November 20, 1997 16:45 Type: N Check: 6409 ..



1 CAGCCCCTCT GGCAGGCTCC CGCCAGCGTC GCTGCGGCTC CGGCCCGGGA

51 GCGAGCGCCC GGAGCTCGGA AAGATGCGGC GCCCGCGGCG GCCTGGGGGT

101 CTCGGGGGAT CCGGGGGTCT CCGGCTGCTC CTCTGCCTCC TGCTGCTGAG

151 CAGCCGCCCG GGGGGCTGCA GCGCCGTTAG TGCCCACGGC TGTCTATTTG

201 ACCGCAGGCT CTGCTCTCAC CTGGAAGTCT GTATTCAGGA TGGCTTGTTT

251 GGGCAGTGCC AGGTGGGAGT GGGGCAGGCC CGGCCCCTTT TGCAAGTCAC

301 CTCCCCAGTT CTCCAACGCT TACAAGGTGT GCTCCGACAA CTCATGTCCC

351 AAGGATTGTC CTGGCACGAT GACCTCACCC AGTATGTGAT CTCTCAGGAG

401 ATGGAGCGCA TCCCCAGGCT TCGCCCCCCA GAGCCCCGTC CAAGGGACAG

451 GTCTGGCTTG GCACCCAAGA GACCTGGTCC TGCTGGAGAG CTGCTTTTAC

501 AGGACATCCC CACTGGCTCC GCCCCTGCTG CCCAGCATCG GCTTCCACAA

551 CCACCAGTGG GCAAAGGTGG AGCTGGGGCC AGCTCCTCTC TGTCCCCTCT

601 GCAGGCTGAG CTGCTCCCGC CTCTCTTGGA GCACCTGCTG CTGCCCCCAC

651 AGCCTCCCCA CCCTTCACTG AGTTACGAAC CTGCCTTGCT GCAGCCCTAC

701 CTGTTCCACC AGTTTGGCTC CCGTGATGGC TCCAGGGTCT CAGAGGGCTC

751 CCCAGGGATG GTCAGTGTCG GCCCCCTGCC CAAGGCTGAA GCCCCTGCCC

801 TCTTCAGCAG AACTGCCTCC AAGGGCATAT TTGGGGACCA CCCTGGCCAC

851 TCCTACGGGG ACCTTCCAGG GCCTTCACCT GCCCAGCTTT TTCAAGACTC

901 TGGGCTGCTC TATCTGGCCC AGGAGTTGCC AGCACCCAGC AGGGCCAGGG

951 TGCCAAGGCT GCCAGAGCAA GGGAGCAGCA GCCGGGCAGA GGACTCCCCA

1001 GAGGGCTATG AGAAGGAAGG ACTAGGGGAT CGTGGAGAGA AGCCTGCTTC

1051 CCCAGCTGTG CAGCCAGATG CGGCTCTGCA GAGGCTGGCC GCTGTGCTGG

1101 CGGGCTATGG GGTAGAGCTG CGTCAGCTGA CCCCTGAGCA GCTCTCCACA

1151 CTCCTGACCC TGCTGCAGCT ACTGCCCAAG GGTGCAGGAA GAAATCCGGG

1201 AGGGGTTGTA AATGTTGGAG CTGATATCAA GAAAACAATG GAGGGGCCGG

1251 TGGAGGGCAG AGACACAGCA GAGCTTCCAG CCCGCACATC CCCCATGCCT

FIG. 3c

1301 GGACACCCCA CTGCCAGCCC TACCTCCAGT GAAGTCCAGC AGGTGCCAAG

1351 CCCTGTCTCC TCTGAGCCTC CCAAAGCTGC CAGACCCCCT GTGACACCTG

1401 TCCTGCTAGA GAAGAAAAGC CCACTGGGCC AGAGCCAGCC CACGGTGGCA

1451 GGACAGCCCT CAGCCCGCCC AGCAGCAGAG GAATATGGCT ACATCGTCAC

1501 TGATCAGAAG CCCCTGAGCC TGGCTGCAGG AGTGAAGCTG CTGGAGATCC

1551 TGGCTGAGCA TGTGCACATG TCCTCAGGCA GCTTCATCAA CATCAGTGTG

1601 GTGGGACCAG CCCTCACCTT CCGCATCCGG CACAATGAGC AGAACCTGTC

1651 TTTGGCTGAT GTGACCCAAC AAGCAGGGCT GGTGAAGTCT GAACTGGAAG

1701 CACAGACAGG GCTCCAAATC TTGCAGACAG GAGTGGGACA GAGGGAGGAG

1751 GCAGCTGCAG TCCTTCCCCA AACTGCGCAC AGCACCTCAC CCATGCGCTC

1801 AGTGCTGCTC ACTCTGGTGG CCCTGGCAGG TGTGGCTGGG CTGCTGGTGG

1851 CTCTGGCTGT GGCTCTGTGT GTGCGGCAGC ATGCGCGGCA GCAAGACAAG

1901 GAGCGCCTGG CAGCCCTGGG GCCTGAGGGG GCCCATGGTG ACACTACCTT

1951 TGAGTACCAG GACCTGTGCC GCCAGCACAT GGCCACGAAG TCCTTGTTCA

2001 ACCGGGCAGA GGGTCCACCG GAGCCTTCAC GGGTGAGCAG TGTGTCCTCC

2051 CAGTTCAGCG ACGCAGCCCA GGCCAGCCCC AGCTCCCACA GCAGCACCCC

2101 GTCCTGGTGC GAGGAGCCGG CCCAAGCCAA CATGGACATC TCCACGGGAC

2151 ACATGATTCT GGCATACATG GAGGATCACC TGCGGAACCG GGACCGCCTT

2201 GCCAAGGAGT GGCAGGCCCT CTGTGCCTAC CAAGCAGAGC CAAACACCTG

2251 TGCCACCGCG CAGGGGGAGG GCAACATCAA AAAGAACCGG CATCCTGACT

2301 TCCTGCCCTA TGACCATGCC CGCATAAAAC TGAAGGTGGA GAGCAGCCCT

2351 TCTCGGAGCG ATTACATCAA CGCCAGCCCC ATTATTGAGC ATGACCCTCG

2401 GATGCCAGCC TACATAGCCA CGCAGGGCCC GCTGTCCCAT ACCATCGCAG

2451 ACTTCTGGCA GATGGTGTGG GAGAGCGGCT GCACCGTCAT CGTCATGCTG

2501 ACCCCGCTGG TGGAGGATGG TGTCAAGCAG TGTGACCGCT ACTGGCCAGA

2551 TGAGGGTGCC TCCCTCTACC ACGTATATGA GGTGAACCTG GTGTCGGAGC

2601 ACATCTGGTG CGAGGACTTT CTGGTGCGGA GCTTCTACCT GAAGAACGTG

2651 CAGACCCAGG AGACGCGCAC GCTCACGCAG TTCCACTTCC TCAGCTGGCC

FIG. 3d

2701 GGCAGAGGGC ACACCGGCCT CCACGCGGCC CCTGCTGGAC TTCCGCAGGA

2751 AGGTGAACAA GTGCTACCGG GGCCGCTCCT GCCCCATCAT CGTGCACTGC

2801 AGTGATGGTG CGGGGAGGAC CGGCACCTAC ATCCTCATCG ACATGGTCCT

2851 GAACCGCATG GCAAAAGGAG TGAAGGAGAT TGACATCGCT GCCACCCTGG

2901 AGCATGTCCG TGACCAGCGG CCTGGCCTTG TCCGCTCTAA GGACCAGTTT

2951 GAATTTGCCC TGACAGCCGT GGCGGAGGAA GTGAATGCCA TCCTCAAGGC

3001 CCTGCCCCAG TGAGACCCTG GGGCCCCTTG GCGGGCAGCC CAGCCTCTGT

3051 CCCTCTTTGC CTGTGTGAGC ATCTCTGTGT ACCCACTCCT CACTGCCCCA

3101 CCAGCCACCT CTTGGGCATG CTCAGCCCTT CCTAGAAGAG TCAGGAAGGG

3151 AAAGCCAGAA GGGGCACGCC TGCCCAGCCT CGCATGCCAG AGCCTGGGGC

3201 ATCCCAGAGC CCAGGGCATC CCATGGGGGT GCTGCAGCCA GGAGGAGAGG

3251 AAAGGACATG GGTAGCAATT CTACCCAGAG CCTTCTCCTG CCTACATTCC

3301 CTGGCCTGGC TCTCCTGTAG CTCTCCTGGG GTTCTGGGAG TTCCCTGAAC

3351 ATCTGTGTGT GTCCCCCTAT GCTCCAGTAT GGAAGAATGG GGTGGAGGGT

3401 CGCCACACCC GGCTCCCCCT GCTTCTCAGC CCCGGGCCTG CCTCTGACTC

3451 ACACTTGGGC GCTCTGCCCT CCCTGGCCTC ACGCCCAGCC TGGTCCCACC

3501 ACCCTCCCAC CATGCGCTGC TCAACCTCTC TCCTTCTGGC GCAAGAGAAC

3551 ATTTCTAGAA AAAACTACTT TTGTACCAGT GTGAATAAAG TTAGTGTGTT

3601 GTCTGTGCAG CTG

FIG. 3e

PREPROINSULIINI
Exon sequences, i.e. sequences to be used in the patent are underlined and represent exon sequences.

V00565 Length: 4992 December 18, 1997 17:50 Type: N Check: 9721 ..





1 CTCGAGGGGC CTAGACATTG CCCTCCAGAG AGAGCACCCA ACACCCTCCA

51 GGCTTGACCG GCCAGGGTGT CCCCTTCCTA CCTTGGAGAG AGCAGCCCCA

101 GGGCATCCTG CAGGGGGTGC TGGGACACCA GCTGGCCTTC AAGGTCTCTG

151 CCTCCCTCCA GCCACCCCAC TACACGCTGC TGGGATCCTG GATCTCAGCT

201 CCCTGGCCGA CAACACTGGC AAACTCCTAC TCATCCACGA AGGCCCTCCT

251 GGGCATGGTG GTCCTTCCCA GCCTGGCAGT CTGTTCCTCA CACACCTTGT

301 TAGTGCCCAG CCCCTGAGGT TGCAGCTGGG GGTGTCTCTG AAGGGCTGTG

351 AGCCCCCAGG AAGCCCTGGG GAAGTGCCTG CCTTGCCTCC CCCCGGCCCT

401 GCCAGCGCCT GGCTCTGCCC TCCTACCTGG GCTCCCCCCA TCCAGCCTCC

451 CTCCCTACAC ACTCCTCTCA AGGAGGCACC CATGTCCTCT CCAGCTGCCG

501 GGCCTCAGAG CACTGTGGCG TCCTGGGGCA GCCACCGCAT GTCCTGCTGT

551 GGCATGGCTC AGGGTGGAAA GGGCGGAAGG GAGGGGTCCT GCAGATAGCT

601 GGTGCCCACT ACCAAACCCG CTCGGGGCAG GAGAGCCAAA GGCTGGGTGT

651 GTGCAGAGCG GCCCCGAGAG GTTCCGAGGC TGAGGCCAGG GTGGGACATA

701 GGGATGCGAG GGGCCGGGGC ACAGGATACT CCAACCTGCC TGCCCCCATG

751 GTCTCATCCT CCTGCTTCTG GGACCTCCTG ATCCTGCCCC TGGTGCTAAG

801 AGGCAGGTAA GGGGCTGCAG GCAGCAGGGC TCGGAGCCCA TGCCCCCTCA

851 CCATGGGTCA GGCTGGACCT CCAGGTGCCT GTTCTGGGGA GCTGGGAGGG

901 CCGGAGGGGT GTACCCCAGG GGCTCAGCCC AGATGACACT ATGGGGGTGA

951 TGGTGTCATG GGACCTGGCC AGGAGAGGGG AGATGGGCTC CCAGAAGAGG

1001 AGTGGGGGCT GAGAGGGTGC CTGGGGGGCC AGGACGGAGC TGGGCCAGTG

1051 CACAGCTTCC CACACCTGCC CACCCCCAGA GTCCTGCCGC CACCCCCAGA

1101 TCACACGGAA GATGAGGTCC GAGTGGCCTG CTGAGGACTT GCTGCTTGTC

1151 CCCAGGTCCC CAGGTCATGC CCTCCTTCTG CCACCCTGGG GAGCTGAGGG

1201 CCTCAGCTGG GGCTGCTGTC CTAAGGCAGG GTGGGAACTA GGCAGCCAGC

1251 AGGGAGGGGA CCCCTCCCTC ACTCCCACTC TCCCACCCCC ACCACCTTGG

1301 CCCATCCATG GCGGCATCTT GGGCCATCCG GGACTGGGGA CAGGGGTCCT

1351 GGGGACAGGG GTCCGGGGAC AGGGTCCTGG GGACAGGGGT GTGGGGACAG

FIG. 3f

1401 GGGTCTGGGG ACAGGGGTGT GGGGACAGGG GTGTGGGGAC AGGGGTCTGG
1451 GGACAGGGGT GTGGGGACAG GGGTCCGGGG ACAGGGGTGT GGGGACAGGG
1501 GTCTGGGGAC AGGGGTGTGG GGACAGGGGT GTGGGGACAG GGGTCTGGGG
1551 ACAGGGGTGT GGGGACAGGG GTCCTGGGGA CAGGGGTGTG GGGACAGGGG
1601 TGTGGGGACA GGGGTGTGGG GACAGGGGTG TGGGGACAGG GGTCCTGGGG
1651 ATAGGGGTGT GGGGACAGGG GTGTGGGGAC AGGGGTCCCG GGGACAGGGG
1701 TGTGGGGACA GGGGTGTGGG GACAGGGGTC CTGGGGACAG GGGTCTGAGG
1751 ACAGGGGTGT GGGCACAGGG GTCCTGGGGA CAGGGGTCCT GGGGACAGGG
1801 GTCCTGGGGA CAGGGGTCTG GGGACAGCAG CGCAAAGAGC CCCGCCCTGC
1851 AGCCTCCAGC TCTCCTGGTC TAATGTGGAA AGTGGCCCAG GTGAGGGCTT
1901 TGCTCTCCTG GAGACATTTG CCCCCAGCTG TGAGCAGGGA CAGGTCTGGC
1951 CACCGGGCCC CTGGTTAAGA CTCTAATGAC CCGCTGGTCC TGAGGAAGAG
2001 GTGCTGACGA CCAAGGAGAT CTTCCCACAG ACCCAGCACC AGGGAAATGG
2051 TCCGGAAATT GCAGCCTCAG CCCCCAGCCA TCTGCCGACC CCCCCACCCC
2101 GCCCTAATGG GCCAGGCGGC AGGGGTTGAC AGGTAGGGGA GATGGGCTCT
2151 GAGACTATAA AGCCAGCGGG GGCCCAGCAG CCCTCAGCCC TCCAGGACAG
2201 GCTGCATCAG AAGAGGCCAT CAAGCAGGTC TGTTCCAAGG GCCTTTGCGT
2251 CAGGTGGGCT CAGGGTTCCA GGGTGGCTGG ACCCCAGGCC CCAGCTCTGC
2301 AGCAGGGAGG ACGTGGCTGG GCTCGTGAAG CATGTGGGGG TGAGCCCAGG
2351 GGCCCCAAGG CAGGGCACCT GGCCTTCAGC CTGCCTCAGC CCTGCCTGTC
2401 TCCCAGATCA CTGTCCTTCT GCCATGGCCC TGTGGATGCG CCTCCTGCCC
2451 CTGCTGGCGC TGCTGGCCCT CTGGGGACCT GACCCAGCCG CAGCCTTTGT
2501 GAACCAACAC CTGTGCGGCT CACACCTGGT GGAAGCTCTC TACCTAGTGT
2551 GCGGGGAACG AGGCTTCTTC TACACACCCA AGACCCGCCG GGAGGCAGAG
2601 GACCTGCAGG GTGAGCCAAC CGCCCATTGC TGCCCCTGGC CGCCCCCAGC
2651 CACCCCCTGC TCCTGGCGCT CCCACCCAGC ATGGGCAGAA GGGGGCAGGA
2701 GGCTGCCACC CAGCAGGGGG TCAGGTGCAC TTTTTTAAAA AGAAGTTCTC
2751 TTGGTCACGT CCTAAAAGTG ACCAGCTCCC TGTGGCCCAG TCAGAATCTC
2801 AGCCTGAGGA CGGTGTTGGC TTCGGCAGCC CCGAGATACA TCAGAGGGTG
2851 GGCACGCTCC TCCCTCCACT CGCCCCTCAA ACAAATGCCC CGCAGCCCAT

FIG. 3g

2901 TTCTCCACCC TCATTTGATG ACCGCAGATT CAAGTGTTTT GTTAAGTAAA
2951 GTCCTGGGTG ACCTGGGGTC ACAGGGTGCC CCACGCTGCC TGCCTCTGGG
3001 CGAACACCCC ATCACGCCCG GAGGAGGGCG TGGCTGCCTG CCTGAGTGGG
3051 CCAGACCCCT GTCGCCAGCC TCACGGCAGC TCCATAGTCA GGAGATGGGG
3101 AAGATGCTGG GGACAGGCCC TGGGGAGAAG TACTGGGATC ACCTGTTCAG
3151 GCTCCCACTG TGACGCTGCC CCGGGGCGGG GGAAGGAGGT GGGACATGTG
3201 GGCGTTGGGG CCTGTAGGTC CACACCCAGT GTGGGTGACC CTCCCTCTAA
3251 CCTGGGTCCA GCCCGGCTGG AGATGGGTGG GAGTGCGACC TAGGGCTGGC
3301 GGGCAGGCGG GCACTGTGTC TCCCTGACTG TGTCCTCCTG TGTCCCTCTG
3351 CCTCGCCGCT GTTCCGGAAC CTGCTCTGCG CGGCACGTCC TGGCAGTGGG
3401 GCAGGTGGAG CTGGGCGGGG GCCCTGGTGC AGGCAGCCTG CAGCCCTTGG
3451 CCCTGGAGGG GTCCCTGCAG AAGCGTGGCA TTGTGGAACA ATGCTGTACC
3501 AGCATCTGCT CCCTCTACCA GCTGGAGAAC TACTGCAACT AGACGCAGCC
3551 TGCAGGCAGC CCCACACCCG CCGCCTCCTG CACCGAGAGA GATGGAATAA
3601 AGCCCTTGAA CCAGCCCTGC TGTGCCGTCT GTGTGTCTTG GGGGCCCTGG
3651 GCCAAGCCCC ACTTCCCGGC ACTGTTGTGA GCCCCTCCCA GCTCTCTCCA
3701 CGCTCTCTGG GTGCCCACAG GTGCCAACGC CAGGCAGGCC CAGCATGCAG
3751 TGGCTCTCCC CAAAGCGGCC ATGCCTGTTG GCTGCCTGCT GCCCCCACCC
3801 TGTGGCTCAG GGTCCAGTAT GGGAGCTTCG GGGGTCTCTG AGGGGCCAGG
3851 GATGGTGGGG CCACTGAGAA GTGACTCTGT CAGTAGCCGA CCTGGAGTCC
3901 CCAGAGACCT TGTTCAGGAA AGGGAATGAG AACATTCCAG CAATTTTCCC
3951 CCCACCTAGC CCTCCCAGGT TCTATTTTTA GAGTTATTTC TGATGGAGTC
4001 CCTGTGGAGG GAGGAGGCTG GGCTGAGGGA GGGGGTCCTG CAGGGCGGGG
4051 GGCTGGGAAG GTGGGGAGAG GCTGCCGAGA GCCACCCGCT ATCCCCAGCT
4101 CTGGGCAGCC CCGGGACAGT CACACACCCT GGCCTCGCGG CCCAAGCTGG
4151 CAGCCGTCTG CAGCCACAGC TTATGCCAGC CCAGGTCCAG CCAGACACCT
4201 GAGGGACCCA CTGGTGCCTT GGAGGAAGCA GGAGAGGTCA GATGGCACCA
4251 TGAGCTGGGG CAGGTGCAGG GACCGTGGCA GCACCTGGCA GGGCCTCAGA
4301 ACCCATGCCT TGGGCACCCC GGCCATGAGG CCCTGAGGAT TGCAGCCCAA
4351 GAGAAGCAGG GAACGCCAGG GCCACAGGGG CAGAGACCAG GCCAGGGTCC

FIG. 3h

4401 CTTGCGGCCC TTAGCCCACC CCCTCCCAGT AAGCAGGGGC TGCTTGGCTA

4451 GGCTTCCTTT TGCTACAGAC CTGCTGCTCA CCCAGAGGCC CACGGGCCCT

4501 AGTGACAAGG TCGTTGTGGC TCCAGGTCCT TGGGGGTCCT GACACAGAGC

4551 CTCTTCTGCA GCACCCCTGA GGACAGGGTG CTCCGCTGGG CACCCAGCCT

4601 AGTGGGCAGA CGAGAACCTA GGGGCTGCCT GGGCCTACTG TGGCCTGGGA

4651 GGTCAGCGGG TGACCCTAGC TACCCTGTGG CTGGGCCAGT CTGCCTGCCA

4701 CCCAGGCCAA ACCAATCTGC ACCTTTCCTG AGAGCTCCAC CCAGGGCTGG

4751 GCTGGGGATG GCTGGGCCTG GGGCTGGCAT GGGCTGTGGC TGCAGACCAC

4801 TGCCAGCTTG GGCCTCGAGG CCAGGAGCTC ACCCTCCAGC TGCCCCGCCT

4851 CCAGAGTGGG GGCCAGGGCT GGGCAGGCGG GTGGACGGCC GGACACTGGC

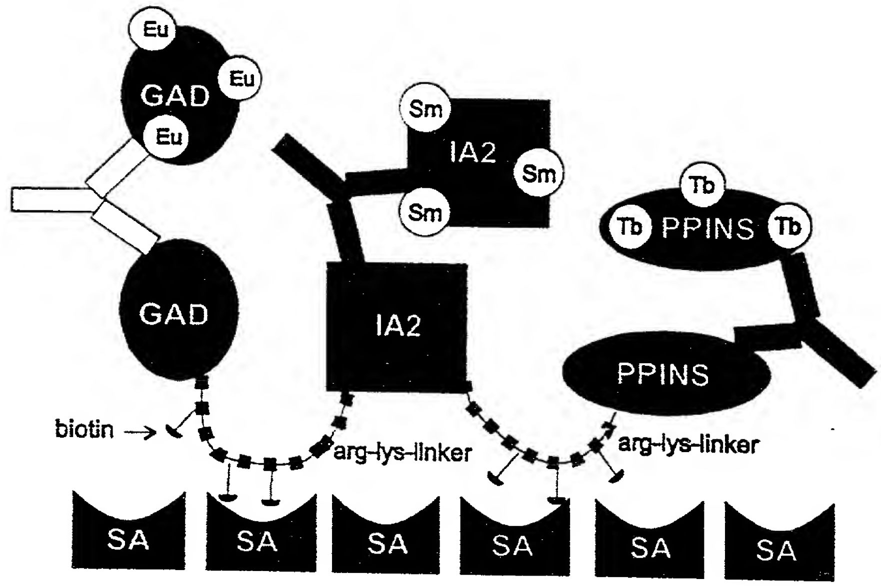
4901 CCCGGAAGAG GAGGGAGGCG GTGGCTGGGA TCGGCAGCAG CCGTCCATGG

4951 GAACACCCAG CCGGCCCCAC TCGCACGGGT AGAGACAGGC GC





FIG. 3i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Eu
GAD
Eu
Sm
IA2
Sm
Sm
Tb
Tb
PPINS
Tb
GAD
IA2
PPINS
biotin
arg-lys-linker
arg-lys-linker
SA
SA
SA
SA
SA
SA

FIG. 4

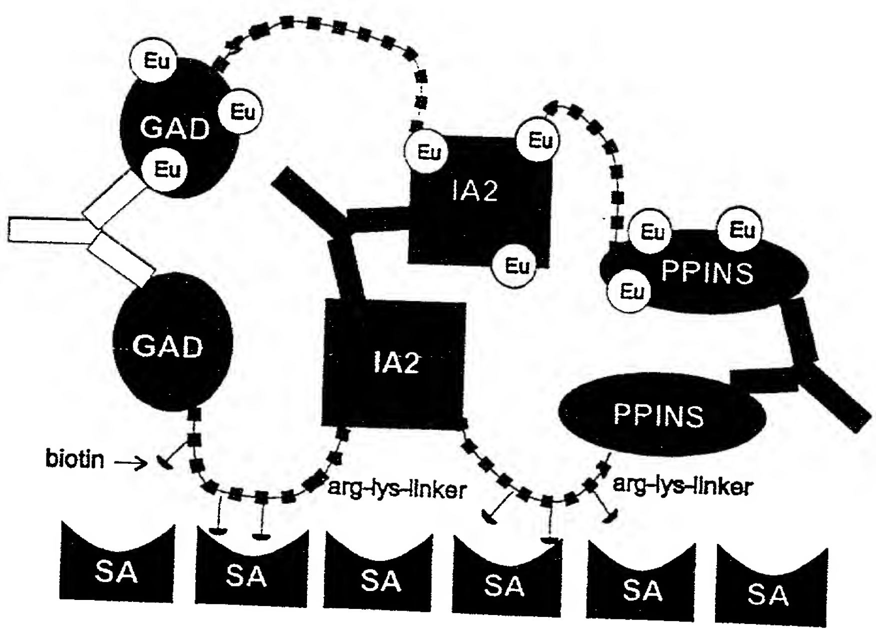
Eu
GAD
Eu
Eu
Eu
Eu
IA2
Eu
Eu
Eu
PPINS
Eu
GAD
IA2
PPINS
biotin
arg-lys-linker
arg-lys-linker
SA
SA
SA
SA
SA
SA

FIG. 5